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Expression and characterization of the p85 subunit of the phosphatidylinositol 3-kinase complex and a related p85β protein by using the baculovirus expression system

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PtdIns 3-kinase associates with certain activated protein-tyrosine kinase receptors and with the pp60$^{c\text{-}src}$/polyoma middle-T complex, suggesting that the enzyme is involved in growth regulation. The purified PtdIns 3-kinase appears to have two subunits, of 85 kDa and 110 kDa. Structural analysis at protein and cDNA levels revealed two forms of the 85 kDa subunit, one which associates with PtdIns 3-kinase activity termed p85α, and a protein of unknown function, p85β. Both 85 kDa proteins contain *src*-homology regions 2 and 3 (SH2 and SH3), but lack enzymic activity, suggesting that they may be regulatory subunits of PtdIns 3-kinase. To probe their structure and function further, p85α and p85β have been expressed and purified in large amounts from insect cells by using baculovirus vectors. Specific antisera detect p85α, but not p85β, associated with PtdIns 3-kinase activity in various cell types. Co-expression studies in insect cells have shown that p85α and p85β are substrates for the protein-tyrosine kinases of epidermal growth factor, colony-stimulating factor 1 and c-*erb*B2 receptors and the *src* family kinase p59$^{c\text{-}fyn}$. Both p85α and p85β form tight complexes with these protein-tyrosine kinases as measured by immunoprecipitation and kinase assays *in vitro*. The specificity of binding of free p85 is less restricted than that of p85 in the active PtdIns 3-kinase complex with the 110 kDa protein. The relevance of these results to growth-factor-induced PtdIns 3-kinase activation is discussed.

INTRODUCTION

A number of structurally diverse polypeptide growth factors act through high-affinity protein-tyrosine kinase receptors, inducing receptor autophosphorylation and phosphorylation of a variety of intracellular targets. Recent evidence from several laboratories suggests that such autophosphorylation triggers the formation of complexes which can involve phospholipase C-γ, PtdIns 3-kinase, GTPase-activating protein, members of the *src* family of cytoplasmic protein-tyrosine kinases, and the serine/threonine kinase Raf (reviewed by Cantley *et al.*, 1991). The fact that these proteins may be involved in the stimulation of important second-messenger systems or in cellular transformation suggests that their association with receptor protein-tyrosine kinases may be of crucial importance in signal transduction.

The association of the PtdIns 3-kinase with activated growth-factor receptors is of particular interest. Increased turnover of PtdIns and its phosphorylated derivatives has been implicated in the action of a number of hormones and growth factors (Berridge & Irvine, 1984). Moreover, altered phosphorylation and turnover of PtdIns have been demonstrated in cells transformed by DNA or RNA viruses (reviewed by Whitman & Cantley, 1988). At least three enzyme activities (type I, II and III), involved in modification of PtdIns by phosphorylation, have been identified in various cell lines and tissues (Whitman *et al.*, 1987; Endemann *et al.*, 1987). The type II and III enzymes are probably both involved in the classical PtdIns pathway and catalyse the formation of PtdIns4*P*. The type I enzyme possesses PtdIns 3-kinase activity (Whitman *et al.*, 1988) and catalyses the formation of PtdIns3*P* and the related species, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which appear to be resistant to cleavage by known phospholipase Cs (Lips *et al.*, 1989; Serunian *et al.*, 1989).

PtdIns 3-kinase activity was first identified in immunoprecipitates of the oncoproteins pp60$^{v\text{-}src}$ (Sugimoto *et al.*, 1984) and pp68$^{v\text{-}ros}$ (Macara *et al.*, 1984). PtdIns 3-kinase activity was subsequently found in immunoprecipitates of polyoma-virus middle-T antigen/pp60$^{c\text{-}src}$ (mT:pp60$^{c\text{-}src}$) complex (Whitman *et al.*, 1985; Courtneidge & Heber, 1987) and receptor protein-tyrosine kinases, including the platelet-derived-growth-factor (PDGF) (Kaplan *et al.*, 1987), colony-stimulating factor 1 (CSF-1) (Varticovoski *et al.*, 1989; Reedijk *et al.*, 1990; Shurtleff *et al.*, 1990), epidermal-growth-factor (EGF) (Bjorge *et al.*, 1990) and insulin receptors (Endemann *et al.*, 1990; Ruderman *et al.*, 1990). Tyrosine phosphorylation of polyoma middle-T antigen or ligand-induced autophosphorylation of receptor molecules has been shown to be very important for their association with PtdIns 3-kinase (Kazlauskas & Cooper, 1989; Talmage *et al.*, 1989). Mutant PDGF receptors or polyoma mT proteins that do not complex with PtdIns 3-kinase lack a full mitogenic response to PDGF or transforming activity respectively (Kaplan *et al.*, 1987; Courtneidge & Heber, 1987; Escobedo & Williams, 1988; Coughlin *et al.*, 1989; Talmage *et al.*, 1989).

Recent studies reveal a possible mechanism involving *src* homology 2 (SH2) domains, by which the formation of multi-enzyme complexes may occur. These SH2 domains, which are approximately 100 amino acids long and contain conserved non-catalytic regions found in a variety of proteins, have been shown to interact specifically with polypeptides containing

Abbreviations used: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CSF-1 colony-stimulating factor 1; FCS, fetal-calf serum.

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phosphotyrosine (Kazlauskas & Cooper, 1989; Kaplan *et al.*, 1990; Mayer *et al.*, 1991; reviewed by Koch *et al.*, 1991). Phospholipase Cγ, GTPase-activating proteins and members of the *src* family all contain SH2 domains (Kaplan *et al.*, 1990; Meisenhelder *et al.*, 1989; Morrison *et al.*, 1989; Kypta *et al.*, 1990). The presence of PtdIns 3-kinase activity in immunoprecipitates, for example, has been closely correlated with the appearance of 81–85 kDa tyrosine-phosphorylated proteins. Recently, we have cloned two bovine brain cDNAs encoding highly related proteins termed p85α and p85β (Otsu *et al.*, 1991). Although these proteins do not possess intrinsic PtdIns 3-kinase activity, p85α is present in a tight complex with a 110 kDa protein which probably encodes the catalytic subunit of the kinase (Otsu *et al.*, 1991; Carpenter *et al.*, 1990; Escobedo *et al.*, 1991; Shibasaki *et al.*, 1991; Hiles *et al.*, 1992). Interestingly, both p85 proteins contain two SH2 domains. They also contain an *N*-terminal (*src* homology region 3) SH3 domain, whose role is less clearly established, but which could mediate interactions with the cytoskeleton (reviewed by Koch *et al.*, 1991), since several cytoskeletal proteins, including myosin 1B (Jung *et al.*, 1987), α-spectrin (Wasenius *et al.*, 1989) and actin-binding protein (Drubin *et al.*, 1990) contain SH3 sequences. The presence of SH2 domains in the p85 proteins suggests that they could be involved in complex-formation with phosphotyrosine-containing proteins, thus acting as adaptors bringing the catalytic domain of the PtdIns 3-kinase to activated protein-tyrosine kinases. This association could in turn activate the enzyme either by subsequent tyrosine phosphorylation or by physical translocation to the plasma membrane, where its substrates are located.

In order to study in detail the biological functions and biochemical properties of p85α and p85β, it was necessary to express them in systems which would allow the purification of large amounts of the recombinant proteins. In this paper, we describe the production of high levels of expressed p85α and p85β protein, using the baculovirus/insect cell expression system. The purification of the expressed proteins to apparent homogeneity by conventional chromatographic techniques is also described. Both p85 proteins are shown to exhibit the properties by which endogenous p85 proteins have been characterized, i.e. both p85α and p85β proteins were shown directly to be substrates for protein-tyrosine kinases and to possess the ability to bind to tyrosine-phosphorylated receptors *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells and viruses

The insect cell line from *Spodoptera frugiperda* (Sf9) was propagated at 27 °C in Grace's medium (Gibco) supplemented with 10% foetal-calf serum (FCS), 3.3 g of lactalbumin hydrolysate (Difco)/l and 3.3 g of TC Yeastolate (Difco)/l. After infection with recombinant virus, cells were grown in IPL-41 medium (Sera Labs) plus 10% FCS, 50 mg of gentamycin (Gibco)/l and 2.5 mg of amphotericin/l. Insect cell culture, identification and purification of recombinant viral clones, and transfection of Sf9 cells were carried out as described previously (Summers & Smith, 1987). All proteins were expressed in Sf9 cells by using baculovirus vectors essentially as described by Greenfield *et al.* (1988).

A pig aortic endothelial cell line stably expressing the human PDGF β-receptor (ZNR) (Claesson-Welsh *et al.*, 1988; Westermark *et al.*, 1990), bovine adrenal-cortex cells (BARC), A431 cells and COS-1 cells were all maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. The pig aortic endothelial cells (ZNR) containing the human PDGF β-receptor were also grown in the presence of 100 μg of G418/ml. If cells were to be stimulated, they were starved in DMEM/0.5% FCS for 36 h. Cells were then stimulated with saturating concentrations of growth factor for 7 min and the immediately washed with phosphate-buffered saline and lysed.

Construction of baculoviral transfer vectors containing p85 cDNA sequences

Since the p85β cDNA sequence contains a unique *Nco*I restriction site near the initiation start codon, the 2.5 kb *Nco*I/*Eco*RI fragment of the p85β cDNA was subcloned into the transfer vector pAcC4 without any modification. p85α cDNA does not contain a *Nco*I site near the ATG codon, so the following procedure was used to clone this sequence into pAcC4. Initially, by using the unique *Kpn*I site 18 bp after the ATG, a 2.22 kb *Kpn*I/*Eco*RI fragment for p85α was subcloned into pAcC4. Two overlapping oligonucleotides, containing a *Kpn*I cloning site on one side and a sticky end for *Nco*I on the other (CATGAGTGCCGAGGGGTAC and CCCTCGGCATC), were generated, annealed together and ligated to *Nco*I/*Kpn*I-digested pAcC4, containing a 2.22 kb *Kpn*I/*Eco*RI fragment for p85α. Several positive pAcC4-p85α clones were sequenced around the ATG, and two of them were used for transfection of Sf9 cells. The subcloned fragments were sequenced by dideoxy chain-termination methods (Sanger *et al.*, 1977) using ^{35}S-dATP.

Transfection of Sf9 cells and isolation of recombinant baculoviruses

Transfection of Sf9 cells was accomplished by the method described by Summers & Smith (1987). Extracellular virions were harvested 2.5 days after transfection. Recombinant viruses were identified by hybridization with p85α or p85β cDNA fragments ^{32}P-labelled by the random-priming method (Feinberg & Volgenstein, 1983) or by identifying polyhedrin-negative plaques under the light microscope (Summers & Smith, 1987).

Infection of Sf9 cells and expression of recombinant proteins

Infections of insect cells with the recombinant viruses were performed in IPL-41 medium supplemented with 10% FCS. Sf9 cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 10. Cells were then harvested and lysed after the appropriate number of days. Co-infections were carried out in the same way, except that a MOI of 5 was used for each recombinant virus and cells were harvested and lysed 3 days after infection. For large-scale expression, 2×10^7 Sf9 cells were infected with recombinant viruses, at a MOI of 10 in 150 cm^2 T-flasks. After 2 days of infection cells were harvested by centrifugation at 1000 *g* for 10 min, washed in phosphate-buffered saline and centrifuged again. Cell pellets were used immediately for purification or immunoprecipitation.

Purification of p85 proteins

Purification of both p85 species was performed by identical methodologies. The Sf9-cell pellet derived from 50 150 cm^2 T-flasks was disrupted on ice, with a Dounce homogenizer, in 40 ml of ice-cold extraction buffer A (20 mM-β-glycerophosphate, pH 7.5, 10 mM-NaF, 10 mM-benzamidine, 2 mM-EDTA, 5 mM-sodium pyrophosphate, 0.2 mM-sodium orthovanadate, 120 mM-NaCl, 0.3% β-mercaptoethanol, 50 μg of phenylmethanesulphonyl fluoride/ml). After 30 min on ice, the lysate was cleared of particulate material by centrifugation at 15000 *g* for 20 min at 4 °C. All subsequent procedures were carried out at 4 °C. The supernatant was loaded on to a 20 ml Q-Sepharose column (Pharmacia) that had been pre-equilibrated in buffer A. The column was washed with 50 ml of buffer A, and bound protein was eluted in 2 ml fractions at 1 ml/min with a linear gradient from 0 to 0.5 M-NaCl in buffer A. All fractions were

assayed by immunoblotting using specific *C*-terminal anti-p85 polyclonal antibodies. Pooled fractions from the Q-Sepharose column (8 ml), containing p85-immunoreactive material, were diluted 5-fold in buffer A and applied to a 2 ml MonoQ column (Pharmacia). After washing in buffer A, protein was eluted in 0.5 ml fractions with a linear NaCl gradient from 0 to 0.4 M. The peak of p85 immunoreactivity was concentrated in a Centriprep concentrator (Amicon) and loaded on a Superose 12 column (Pharmacia) equilibrated in buffer A containing 0.2 M-NaCl. Eluted fractions containing p85 were pooled and concentrated in a Centriprep concentrator. Purified material was dialysed against 20 mM-Hepes (pH 7.0)/150 mM-NaCl/50% (v/v) glycerol and then stored at −20 °C. Under these conditions p85 proteins remained active, as measured by their ability to associate with tyrosine-phosphorylated proteins, for over a year.

Immunological methods

Preparative SDS/PAGE was used to purify p85α and p85β from Sf9 cells infected with the recombinant virus. Purified protein (100 mg) was mixed with Freund's adjuvant and subcutaneously injected into two rabbits. The animals were boosted every 4 weeks and serum was collected after 10 days. Serum was tested after each boost by immunoprecipitation and Western blotting. After the fifth boost, the sera were affinity-purified by using p85 proteins coupled to Actigel-ALD-Superflow resin (Sterogene). A 2 mg portion of purified p85 was coupled to 1 ml of Actigel. Crude serum was incubated with the coupled protein at 4 °C overnight and was eluted from the Actigel according to the manufacturer's recommendations.

The *C*-terminal peptide antisera were prepared against the bovine *C*-terminal sequences determined by cDNA cloning (Otsu *et al.*, 1991). The peptide sequences TLAYPVYAQQRR for p85α and TLAHPVRAPGPGPPPAAR for p85β were synthesized by 9-fluorenylmethoxycarbonyl (FMOC) chemistry and purified by h.p.l.c. The peptides were then coupled to keyhole-limpet haemocyanin and injected into rabbits. Antisera scoring positive by e.l.i.s.a. were affinity-purified on peptide affinity columns.

Monoclonal antibody 327 (Oncogene Science Inc.) was used to immunoprecipitate $pp60^{c\text{-}src}$ (Lipsich *et al.*, 1983). The R1 monoclonal antibody against the human EGF receptor was raised as described (Waterfield *et al.*, 1982). The monoclonal antibody (clone 3-4A4-E4) against the CSF-1 receptor is described by Ashmun *et al.* (1989). The peptide antiserum against $p59^{c\text{-}fyn}$ is described by Kypta *et al.* (1988). The PR4 antiserum against the *C*-terminal 13 amino acids of the PDGF receptor was raised as described by Kypta *et al.* (1990). The anti-phosphotyrosine antibody used was raised against Ala-Gly-phosphoTyr-KLH as described previously by Kamps & Sefton (1988) (M. J. Fry & G. Panayotou, unpublished work).

Purification from bovine brain and assay of PtdIns 3-kinase

PtdIns 3-kinase was purified from bovine brain as described by Morgan *et al.* (1990). Its activity was determined essentially as described by Whitman *et al.* (1985).

Immunoprecipitation of PtdIns 3-kinase activity from cell lines and partially purified bovine brain preparation

For 3-kinase analysis, lysates were prepared of near-confluent cells in EB lysis buffer (20 mM-Tris, pH 7.4, 50 mM-NaCl, 50 mM-NaF, 1% Nonidet P-40, 1 mM-EDTA, 500 μM-sodium orthovanadate, 2 mM-phenylmethanesulphonyl fluoride, 100 kallikrein-inhibitor units of aprotinin/ml) (Kazlauskas & Cooper, 1988). After collection of immune complexes on Protein A–Sepharose beads, the samples were either subjected to PtdIns 3-kinase assays or phosphorylated *in vitro*. This involved extensive washing; twice with EB lysis buffer and twice with kinase buffer (50 mM-Hepes, pH 7.4, 150 mM-NaCl, 0.02% Triton X-100, 12 mM-$MgCl_2$, 1 mM-$MnCl_2$, 10% glycerol, 500 μM-sodium orthovanadate). The complexes were phosphorylated in the presence of 10 μCi of [γ-^{32}P]ATP, and phosphorylated proteins were analysed by SDS/PAGE.

Immunoprecipitation of PtdIns 3-kinase activity from the partially purified bovine brain preparation was performed in essentially the same way. PtdIns 3-kinase was diluted in EB buffer, immunoprecipitated, and immune complexes were subjected to PtdIns 3-kinase assays.

Association of receptor kinases with p85α, p85β and bovine brain PtdIns 3-kinase

Sf9 cells were co-infected as described above. Then 3 days after infection the cells were either lysed and boiled in 2 × SDS/PAGE sample buffer and then subjected to Western-blot analysis with an affinity-purified anti-phosphotyrosine antibody performed as described by Kamps & Sefton (1988), or lysed in lysis buffer (50 mM-Tris, pH 7.4, 150 mM-NaCl, 1% Triton X-100, 2 mM-phenylmethanesulphonyl fluoride, 100 kallikrein-inhibitor units of aprotinin/ml, 500 μM-sodium orthovanadate). Lysates prepared from cells co-infected with recombinant baculovirus vectors expressing both a protein-tyrosine kinase and one of the p85 proteins were incubated with antibodies specific for the appropriate protein-tyrosine kinase. After collecting the immune complexes on Protein A–Sepharose beads and extensive washing (twice with lysis buffer, twice with kinase buffer), the immune complexes were phosphorylated in the presence of 5 μCi of [γ-^{32}P]ATP and phosphorylated proteins were analysed by SDS/PAGE.

The assay for the blocking of association *in vitro* of the CSF-1 receptor to the partially purified bovine brain preparation by baculovirus-purified p85 proteins was performed as essentially described by Kazlauskas & Cooper (1990). The CSF-1 receptor was immunoprecipitated from Sf9 cells and collected on Protein A–Sepharose beads. After extensive washing (three times with lysis buffer, twice with kinase buffer), the receptor was phosphorylated for 15 min at 20 °C with non-labelled ATP. The immunoprecipitates were then washed again to remove free ATP and then incubated with p85α, p85β, PtdIns 3-kinase, or PtdIns 3-kinase together with various amounts of p85α or p85β proteins respectively for 2 h at 4 °C. The immune complexes were then washed again and then either subjected to phosphorylation in the presence of 5 mCi of [γ-^{32}P]ATP or assayed for associated PtdIns 3-kinase activity. Phosphorylated proteins were analysed by SDS/PAGE, and phospholipids were analysed by t.l.c. (Whitman *et al.*, 1985).

RESULTS

Expression of p85α and p85β in insect cells

Expression of cDNAs in insect cells using baculovirus vectors often provides a very efficient means of obtaining large quantities of a protein. The coding region of a cDNA is placed under the control of the powerful polyhedrin promoter, in some cases allowing high levels of expression (Cameron *et al.*, 1989; Luckow & Summers, 1988). Both p85 cDNAs were subcloned into the pAcC4 baculovirus transfer vector so that the initiating methionine residues of the p85 coding sequences were introduced downstream of the polyhedrin promoter. These techniques have been briefly described previously for p85β (Otsu *et al.*, 1991). The

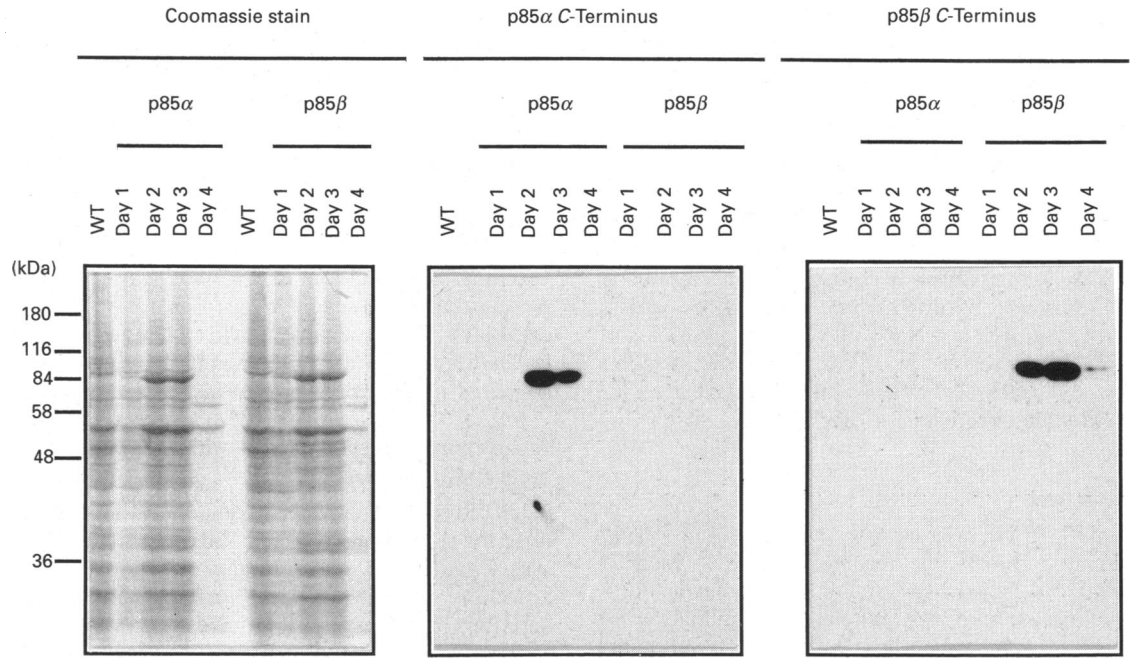

Fig. 1. Expression of p85α and p85β in Sf9 cells

Sf9 cells were infected with either wild-type (WT) or recombinant baculovirus vectors expressing p85α or p85β. WT-baculovirus-infected cells were harvested after 2 days, and cells infected with recombinant viruses were harvested at the times indicated at the top of each panel. Lysates were subjected to SDS/PAGE in 10% gels and then analysed as follows. Left panel: Coomassie Blue stain of lysates from Sf9 cells infected with recombinant baculovirus vectors. Middle panel: immunoblot of Sf9-cell lysates probed with *C*-terminal p85α-specific antiserum. Right panel: immunoblot of Sf9-cell lysates probed with *C*-terminal p85β-specific antiserum.

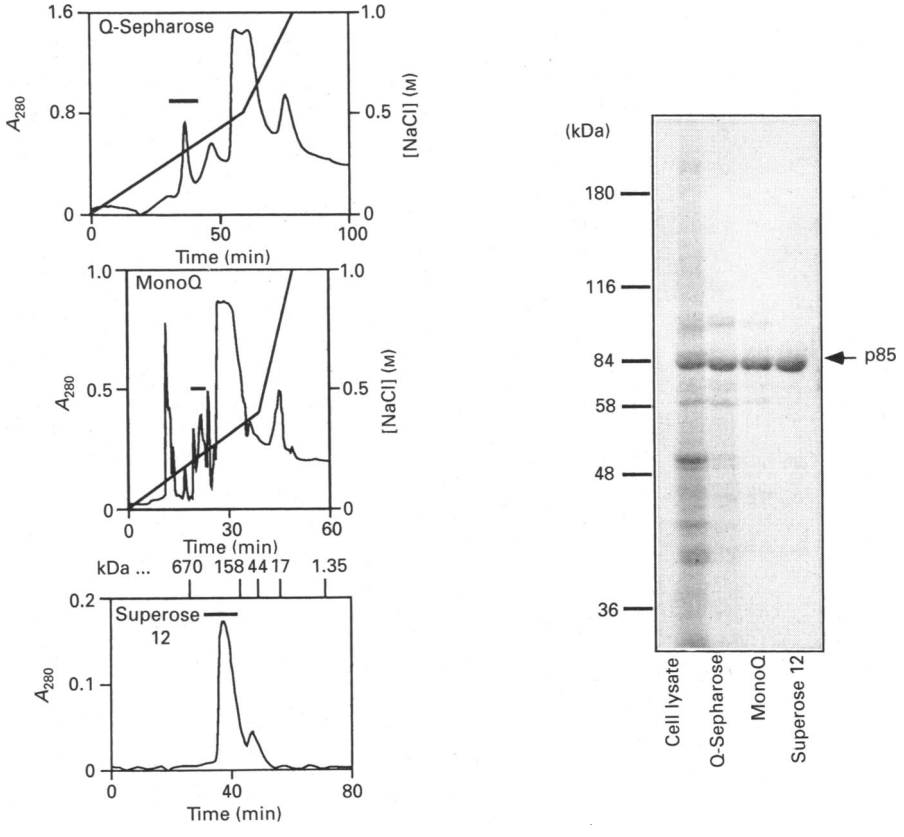

Fig. 2. Purification of p85 proteins

Typical column profiles from a representative purification of p85α are shown. The bars above each trace show the fractions containing p85 proteins eluted from each column which were then pooled. Top left panel: profile of Q-Sepharose column. Middle left panel: profile of MonoQ f.p.l.c. column. Bottom left panel: profile of Superose 12 f.p.l.c. column. Right panel: Coomassie-Blue-stained gel of total Sf9-cell lysates and samples from pooled peak fractions throughout a typical purification of p85α.

recombinant viruses expressing p85α and p85β were used to infect cultures of the insect cell line, Sf9. The levels of p85 protein expression were monitored by analysing whole cell lysates by SDS/PAGE at various times after infection. Fig. 1 (left panel) shows results which indicate that, within 2 days, high levels of both p85α and p85β proteins begin to accumulate inside the insect cells. Maximal expression was observed between days 2 and 3 after infection, at which point p85 proteins accounted for up to 10% of total cell protein, and then decreased as the virus entered into its lytic phase. Probing with specific affinity-purified *C*-terminal peptide antibodies in Western-blot assays further confirmed the identity of the proteins [Fig. 1, middle (p85α) and right (p85β) panels]. These antibodies were specifically reacting only with the p85 produced in cells infected with the p85α (Fig. 1, middle panel) or p85β (Fig. 1, right panel) recombinant viruses respectively. Cells infected with control wild-type virus alone showed no detectable immunoreactive protein 2 days after infection.

Purification of baculovirus-expressed p85α and p85β

Both p85 proteins could be isolated by identical purification schemes, reflecting the high degree of similarity between them. Briefly, the purification protocol adopted is as follows. Stocks of recombinant virus were used to infect approx. 5×10^9 cells grown in monolayer cultures in 50 150 cm^2 flasks. At 2 days after infection, cells were harvested by centrifugation and washed with phosphate-buffered saline. The solubility of the two proteins during cell extraction was tested. Cells were either homogenized with a Dounce homogenizer in the absence of detergents or lysed in the presence of 1% Triton X-100. After centrifugation, the clarified supernatants and the insoluble pellets were analysed by SDS/PAGE and the p85 proteins were identified by Coomassie Blue staining. Both p85 proteins were found to be readily extractable from cells in the absence of detergents. This is in agreement with previous reports, which have suggested that in unstimulated cells the bulk of the p85 is cytoplasmic (Cohen *et al.*, 1990). The soluble cell lysate was applied to a 20 ml Q-Sepharose (anion-exchange) column, and bound p85 was eluted at a NaCl concentration of 200–250 mM (Fig. 2, top left panel). Further purification and concentration were achieved by applying the pooled peak fractions to a MonoQ f.p.l.c. column (Fig. 2, middle left panel). Both p85 proteins were eluted from this column at approx. 200 mM salt. This material was then subjected to gel-exclusion chromatography on a Superose 12 f.p.l.c. column (Fig. 2, bottom left panel). Analysis of a typical purification for p85α is shown in Fig. 2. Both p85 proteins exhibited identical chromatographic behaviour on these columns. The p85α and p85β proteins were eluted from the Superose 12 column at a position suggesting that they had an apparent molecular mass of approx. 210 kDa. It is unclear at the moment whether this is due to formation of p85 dimers. Fig. 2 (right panel) shows SDS/PAGE analysis of the material applied to the various columns and the purified product. No significant further purification was possible by a highly specific affinity-purification step described in Otsu *et al.* (1991), based on the interaction of p85 with a tyrosine-phosphorylated synthetic peptide corresponding to the kinase insert region of the PDGF receptor (results not shown). The overall yield of pure p85 was approx. 50%, resulting in approx. 10 mg of protein purified from 5×10^9 infected Sf9 cells.

Preparation and characterization of antisera against p85 proteins

Antisera against p85α and p85β have been raised in rabbits against purified native p85 proteins as well as against SDS/PAGE-purified denatured p85 proteins. The data presented here were all generated with the antisera raised against denatured p85 proteins, but essentially identical results have been obtained with the antisera raised against native p85 proteins. By immunoprecipitation analysis, these antibodies predominantly recognized the p85 species against which they were raised (Fig. 3). The two antisera were affinity-purified by using p85α– and p85β–Actigel columns, and their ability to immunoprecipitate specifically recombinant p85 proteins from Sf9-cell lysates was tested. As shown in Fig. 3, despite the high degree of similarity between the two p85 proteins (Otsu *et al.*, 1991), these antibodies showed a high degree of specificity in their ability to immunoprecipitate the appropriate p85 protein. These antisera were then used to immunoprecipitate PtdIns 3-kinase activity from the partially purified bovine brain preparation. As shown in Fig. 4, despite the high titre of both of these antisera only the p85α antibody immunoprecipitated significant amounts of bovine

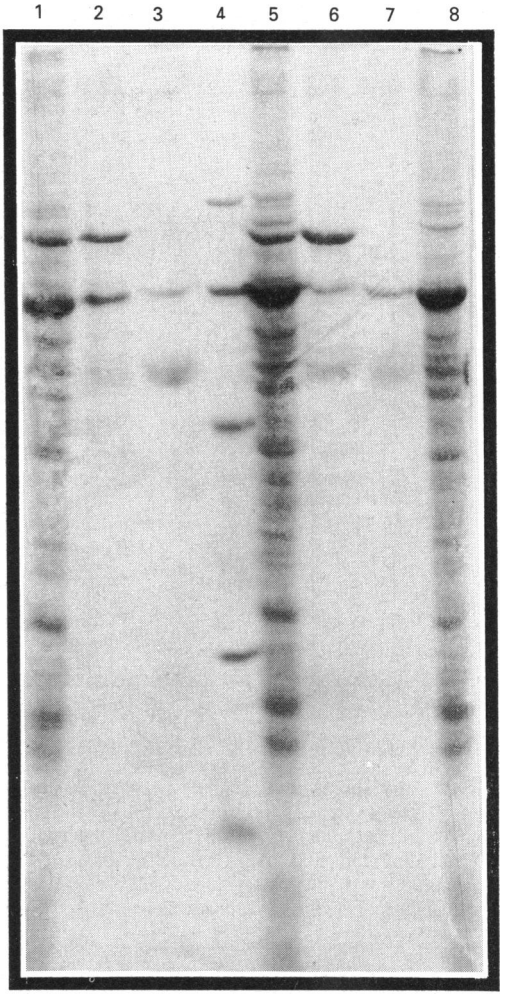

Fig. 3. Characterization of polyclonal antibody against p85α and p85β

The specificity of the affinity-purified p85α and p85β antibodies to the proteins to which they were raised was examined. The antibodies were incubated with lysates of Sf9 cells expressing p85α and p85β proteins, and the immunocomplexes were then subjected to SDS/PAGE. Lanes: 1, crude lysate of Sf9 cells expressing p85β protein; 2, p85β-expressing Sf9 cells immunoprecipitated with p85β antibody; 3, p85β-expressing Sf9 cells immunoprecipitated with p85α antibody; 4, molecular-mass standards (97, 68, 44, 30, 21 kDa); 5, crude lysate of Sf9 cells expressing p85α protein; 6, p85α-expressing Sf9 cells immunoprecipitated with p85α antibody; 7, p85α-expressing Sf9 cells immunoprecipitated with p85β antibody; 8, crude lysate of WT-infected Sf9 cells.

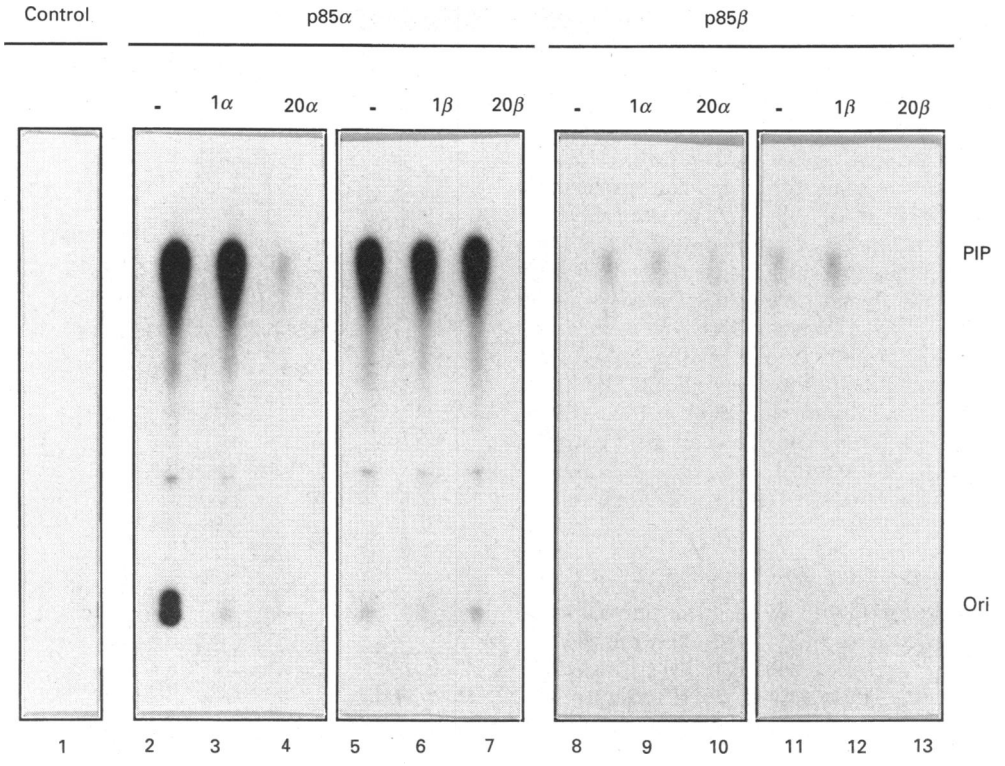

Fig. 4. Immunoprecipitation of PtdIns 3-kinase activity from the partially purified bovine brain preparation using affinity-purified polyclonal p85 antibodies

Polyclonal p85 antibodies were incubated with partially purified bovine brain preparation diluted in EB buffer with or without baculovirus-purified p85 proteins. These immunocomplexes were then used in a PtdIns 3-kinase assay. Lanes: 2, 5, 8 and 11, PtdIns 3-kinase in EB buffer; 3 and 9, PtdIns 3-kinase in EB buffer + 1 μg of p85α; 4 and 10, PtdIns 3-kinase in EB buffer + 20 μg of p85α; 5 and 11, PtdIns 3-kinase in EB buffer + 1 μg of p85β; 7 and 13, PtdIns 3-kinase in EB buffer + 20 μg of p85β. Lanes 2–7 were immunoprecipitated with anti-p85α serum, and lanes 8–13 with anti-p85β serum. Lane 1: PtdIns 3-kinase in EB buffer incubated with Protein A–Sepharose alone. 'Ori' and 'PIP' markers indicate the positions of the t.l.c. origin and PtdIns3*P* respectively.

brain PtdIns 3-kinase activity. The small amounts of PtdIns 3-kinase activity observed to be immunoprecipitated by the p85β antibody (< 1 % of the amount of activity precipitated by p85α antisera) is possibly due to weak cross-reactivity of these antisera, as it has been shown by immunoblotting with the *C*-terminal-specific peptide antibodies that the purified bovine brain preparation contains no detectable p85β protein (Fry *et al.*, 1992). However, we also consider it possible that PtdIns 3-kinase activity associated with the p85β protein might also exist and be present at a low level in the bovine brain preparation. To characterize these antisera further, the ability of increasing concentrations of baculovirus-expressed recombinant p85 proteins to compete with the bovine brain PtdIns 3-kinase in an immunoprecipitation assay with the p85α-specific antisera was examined. Purified baculovirus p85α protein was observed to block the immunoprecipitation of PtdIns 3-kinase activity from bovine brain, but no competition was observed even at the highest concentrations of added purified baculovirus p85β protein (Fig. 4). It should be noted, however, that the small amount of PtdIns 3-kinase activity immunoprecipitated with the p85β antiserum was also specifically competed with purified baculovirus p85β, but not p85α, protein. This result strengthens the above suggestion that there may be a small population of p85β-associated PtdIns 3-kinase activity present in the bovine brain purified PtdIns 3-kinase preparation.

The affinity-purified p85α antibody was found to immunoprecipitate PtdIns 3-kinase activity from a wide range of species, including human (A431), bovine (BARC), simian (COS-1), murine (NIH 3T3) and pig (ZNR) cells (Fig. 5). However, little or no PtdIns 3-kinase activity was detected in any of these cell lines after immunoprecipitation with the affinity-purified p85β antibody (Fig. 5). Immunoblotting of these cell lines with specific peptide antisera shows that the predominant p85 species in all of these lines is p85α, with p85β protein being present at less than 10 % the level of p85α (results not shown).

The effects of growth-factor stimulation on total cellular PtdIns 3-kinase activity was also examined in one of these cell lines (ZNR), which expresses the human PDGF β-receptor. Cells were grown to confluence and then starved for 36 h in 0.5 % FCS to bring them to quiescence. Half of the dishes were then stimulated for 7 min with PDGF-BB and then the cells were lysed. The cell lysates were immunoprecipitated with an excess of the respective antibodies as determined by previous titration experiments (results not shown). A significant increase in PtdIns 3-kinase activity (up to 50-fold) was found associated with the PDGF receptor after ligand stimulation (Fig. 6, lanes 5 and 6). However, immunoprecipitation with the p85α antiserum showed that only a small increase (2–3-fold) in PtdIns 3-kinase activity was detected present in PDGF-stimulated ZNR cells relative to the unstimulated cells (Fig. 6, lanes 3 and 4).

Expressed p85 proteins are substrates for protein-tyrosine kinases

In order to investigate the interaction between the expressed p85α and p85β and a variety of growth-factor receptors encoding protein-tyrosine kinases, these molecules were co-expressed in insect cells by using recombinant viruses. The receptor molecules employed were the EGF receptor, the CSF-1 receptor, and the c-*erb*B2 protein, which encodes an EGF-related receptor for an uncharacterized growth factor. The non-receptor protein-tyrosine kinase $p59^{c\text{-}fyn}$ was also used in these assays. The protein-tyrosine kinases were expressed in insect cells either alone or

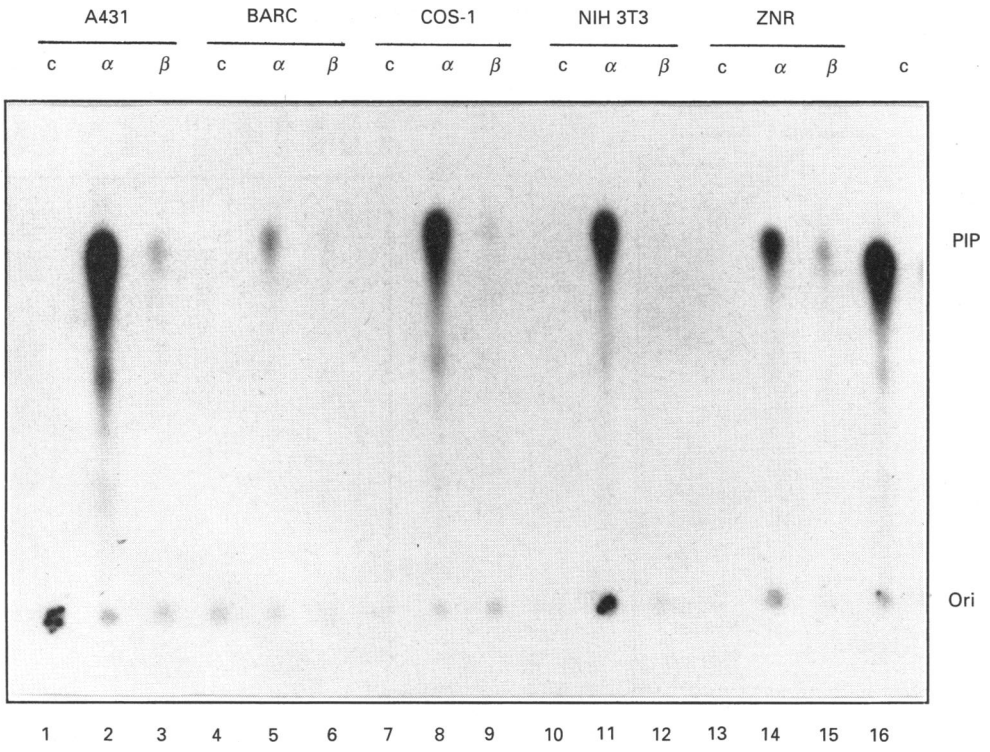

Fig. 5. Immunoprecipitation of PtdIns 3-kinase activity from cell lines

Confluent A431, BARC, COS-1, NIH 3T3 and ZNR cells were lysed and immunoprecipitated with affinity-purified anti-p85α (α) or p85β (β) antibodies or Protein A–Sepharose alone (c). PtdIns 3-kinase assays were then performed on the samples. Lanes 1, 4, 7, 10 and 13 were precipitated with Protein A–Sepharose alone, lanes 2, 5, 8, 11 and 14 with anti-p85α serum, and lanes 3, 6, 9, 12 and 15 with anti-p85β serum. Lane 16: PtdIns 3-kinase activity in 0.5 μl of partially purified bovine brain preparation. 'Ori' and 'PIP' markers indicate the positions of the t.l.c. origin and migration of PtdIns3*P*.

together with p85α or p85β. Cell lysates from infected cells were subjected to SDS/PAGE and immunoblotting with an affinity-purified anti-phosphotyrosine antibody. As shown in Fig. 7, both p85α and p85β were highly phosphorylated on tyrosine residues when co-expressed with any of the protein-tyrosine kinases, but not when expressed on their own.

A tight complex is formed between the p85 proteins and protein-tyrosine kinases

The results described above demonstrate that both p85 proteins are substrates *in vivo* for a variety of protein-tyrosine kinases. It has been previously demonstrated that the p85 proteins can also associate with certain protein-kinases *in vitro* (Kazlauskas & Cooper, 1988, 1989; Otsu *et al.*, 1991). That the p85 proteins can also form stable complexes with these various protein-tyrosine kinases was demonstrated by immunoprecipitation and by kinase experiments performed *in vitro* using co-infected Sf9 cells. Lysates of cells, co-infected with two recombinant baculovirus vectors expressing a protein-tyrosine kinase and one of the p85 species, were incubated with antibodies specific for the appropriate protein-tyrosine kinase, and the immune complexes were collected on Protein A–Sepharose beads. After several washes in lysis buffer, the beads were incubated with a kinase assay mixture containing radiolabelled ATP, and phosphorylated proteins were analysed by SDS/PAGE and autoradiography. Fig. 8 shows that both p85α and p85β formed tight complexes with, and were phosphorylated by, the protein-tyrosine kinase receptors for EGF and CSF-1, and by the *src*-family protein-tyrosine kinase, $p59^{c\text{-}fyn}$. Preincubation of the infected cells with specific growth factors did not have any effect on the observed association and phosphorylation events, as most receptors expressed by using the baculovirus system appear to be constitutively activated (results not shown).

Blocking of binding of bovine brain PtdIns 3-kinase to CSF-1 receptor with recombinant p85 proteins

The finding that the p85 proteins interact with protein-tyrosine kinases prompted us to investigate the ability of recombinant p85 proteins to block binding of the partially purified bovine brain PtdIns 3-kinase activity to the CSF-1 receptor. The assay used to examine this is a modification of the phosphotyrosine-dependent binding assay for substrate proteins *in vitro* to PDGF receptor originally described by Kazlauskas & Cooper (1988). CSF-1 receptor was expressed in Sf9 cells by using recombinant baculovirus vectors. At 2 days post-infection the cells were lysed and the receptor was immunoprecipitated with anti-(CSF-1 receptor) antibody. To form receptor complexes *in vitro*, the receptor was phosphorylated with non-radiolabelled ATP. The remaining unused ATP was removed by washing, and then the immunoprecipitates were incubated for 2 h with purified p85α or p85β, or with bovine brain PtdIns 3-kinase alone, or with bovine brain PtdIns 3-kinase together with increasing concentrations of either p85α or p85β. After extensive washing, immunocomplexes were subjected to phosphorylation *in vitro* in the presence of radiolabelled ATP or were assayed for associated PtdIns 3-kinase activity. As shown in Fig. 9, bovine brain PtdIns 3-kinase activity (Fig. 9*b*) and an 85 kDa phosphoprotein (Fig. 9*a*) from this preparation bind to the CSF-1 receptor. As the concentration of either of the recombinant p85 proteins added to the receptor *in vitro* is increased, the amount of PtdIns 3-kinase activity bound to the receptor is observed to decrease. At high

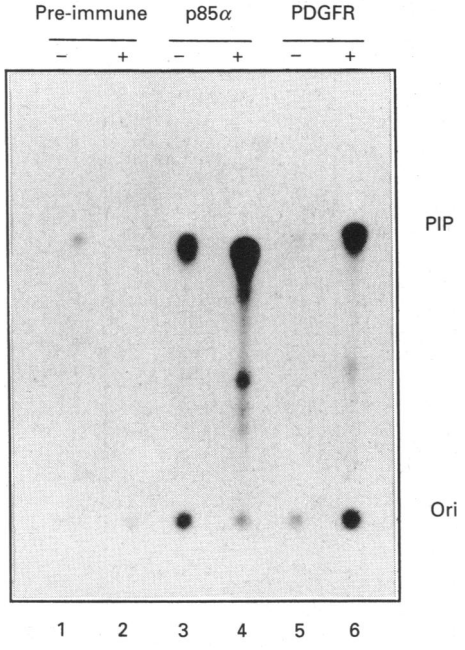

Fig. 6. Immunoprecipitation of PtdIns 3-kinase activity from PDGF-stimulated and unstimulated ZNR cells

Confluent quiescent pig aortic endothelial (ZNR) cells expressing the human PDGF β-receptor were stimulated with recombinant PDGF-BB (+; lanes 2, 4 and 6) or left unstimulated (−; lanes 1, 3 and 5). Lysates were prepared and then immunoprecipitated with pre-immune rabbit serum (lanes 1 and 2), p85α antiserum (lanes 3 and 4) or PR4 antiserum (PDGFR; lanes 5 and 6), and subjected to PtdIns 3-kinase assay. 'Ori' and 'PIP' markers indicate the positions of the t.l.c. origin and PtdIns3*P* respectively.

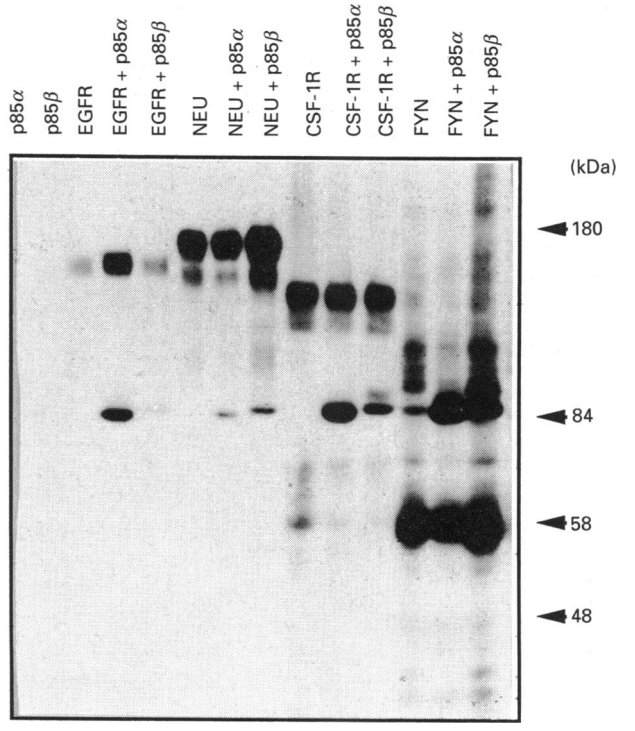

Fig. 7. Phosphorylation of p85 proteins *in vivo* by protein-tyrosine kinases

Co-expression of p85 proteins together with protein-tyrosine kinases in Sf9 cells analysed by Western-blot analysis with an affinity-purified anti-phosphotyrosine antibody in accordance with Kamps & Sefton (1988). Lanes: 1 and 2, crude lysate of Sf9 cells expressing p85α and p85β proteins alone; 3–5, crude lysates of Sf9 cells expressing EGF receptor (EGFR) alone or with p85α or p85β proteins respectively; 6–8, crude lysates of Sf9 cells expressing c-*erb*B2 receptor alone or with p85α or p85β proteins respectively; 9–11, crude lysates of Sf9 cells expressing CSF-1 receptor (CSF-1R) alone or with p85α or p85β proteins respectively; 12–14, crude lysates of Sf9 cells expressing $p59^{c\text{-}fyn}$ receptor (FYN) alone or with p85α or p85β proteins respectively.

concentrations both p85α and p85β completely inhibit binding of bovine brain PtdIns 3-kinase activity to this receptor (Fig. 9*a*).

DISCUSSION

Recent evidence from several laboratories has highlighted the potential importance of PtdIns 3-kinase activation in growth-factor-mediated mitogenesis as well as in transformation by oncogenes (reviewed by Whitman & Cantley, 1988; Cantley *et al.*, 1991). The structure of the catalytic domain of the PtdIns 3-kinase remains unknown. We have recently cloned two related proteins, p85α and p85β. Although they are not catalytic themselves, one of these proteins, p85α, is found in a complex with the putative 110 kDa catalytic subunit of the PtdIns 3-kinase (Otsu *et al.*, 1991). A similar conclusion regarding the composition of a major form of active PtdIns 3-kinase complex has recently been reached by two other laboratories, who purified PtdIns 3-kinase from rat liver and bovine thymus respectively (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991). The presence of SH2 domains in these p85 proteins suggested that they may mediate the association of the PtdIns 3-kinase to activated autophosphorylated receptors. Indeed, our preliminary observations showed that p85α and p85β formed complexes with, and were phosphorylated by, the EGF and PDGF receptors, as well as with the polyoma middle-T/$pp60^{c\text{-}src}$ complex (Otsu *et al.*, 1991). Here we extend these findings to other receptor and non-receptor protein-tyrosine kinases and study their interactions with both expressed p85α and p85β.

We have expressed the two cloned p85 proteins in insect cells using recombinant baculovirus vectors, since it is well established that this system results in high-level protein expression (Cameron *et al.*, 1989; Luckow & Summers, 1988). In constructing the expression vectors we decided not to include a signal sequence for secretion of these proteins, although that would have facilitated purification from conditioned media. The fact that the p85 proteins are normally cytosolic could, in our experience, present problems with their efficient secretion. Both proteins were readily extracted from the cells in the absence of detergents, and their high levels of expression resulted in a simple and efficient purification protocol. The isolation of milligram amounts of the two p85 proteins has allowed us to begin the characterization of their three-dimensional structure by crystallographic and other techniques. Moreover, localization of these p85 proteins in the cytoplasm has also allowed the investigation of their interactions with protein-tyrosine kinases, as these molecules can be easily co-expressed in the same cells.

The identification of specific substrates for protein-tyrosine kinases has proved very difficult to date, owing to their low abundance in the cell and the difficulty in distinguishing between physiologically relevant and fortuitous phosphorylation events. Recent findings have focused attention on a number of important enzymes thought to be involved in second-messenger production and the regulation of intracellular signalling (e.g. phospholipase C-γ, GTPase-activating protein etc.) as potential substrates for

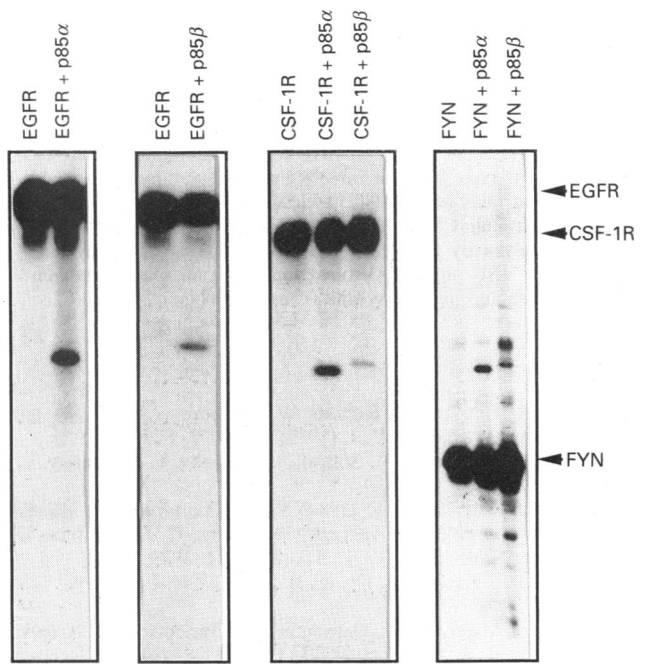

Fig. 8. Association with and phosphorylation *in vitro* of p85 proteins by protein-tyrosine kinases

Lysates prepared from Sf9 cells infected with recombinant baculovirus virus expressing EGF receptor (EGFR), CSF-1 receptor (CSF-1R) or $p59^{c\text{-}fyn}$ (FYN) protein-tyrosine kinase alone, or with one of the p85 proteins, as indicated, were incubated with appropriate protein kinase-specific antibodies, and the immune complexes were then radiolabelled in the presence of [γ-^{32}P]ATP, resolved by SDS/PAGE on 7.5% gels, and finally subjected to autoradiography. Migration positions of protein-tyrosine kinases are marked by arrowheads.

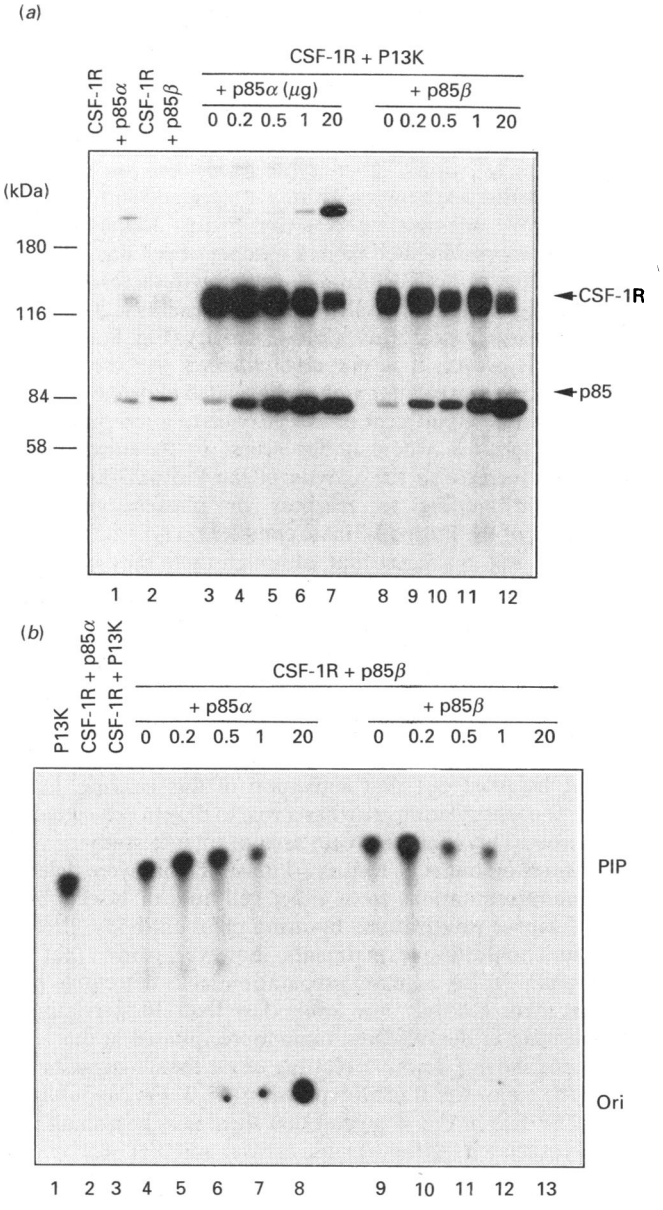

Fig. 9. Blocking of association *in vitro* of bovine brain PtdIns 3-kinase with CSF-1 receptor by baculovirus p85α and p85β proteins

Immunoprecipitated baculovirus-expressed CSF-1 receptor (CSF-1R) was pre-phosphorylated by incubation with non-labelled ATP and then exposed to 1 μg of baculovirus-purified p85α or p85β proteins (lanes 1 and 2 respectively), bovine brain purified PtdIns 3-kinase (PI3K) diluted in lysis buffer (lane 3), bovine brain purified PtdIns 3-kinase and increasing amounts of p85α protein (0, 0.2, 0.5, 1 and 20 μg; lanes 3–7 respectively), bovine brain purified PtdIns 3-kinase and increasing amounts of p85β protein (0, 0.2, 0.5, 1 and 20 μg; lanes 8–12 respectively). To detect the associated proteins, the samples were divided into two. Half was subjected to protein kinase assay *in vitro* (*a*), analysed by SDS/PAGE and detected by autoradiography. The other half was used to measure receptor-associated PtdIns 3-kinase activity. The phosphorylated lipids were separated by t.l.c. and detected by autoradiography. Lanes 2–13 in (*b*) are the equivalent samples in lanes 1–12 in (*a*) respectively. Lane 1 in (*b*) is the amount of PtdIns 3-kinase used in the assay. 'Ori' and 'PIP' markers indicate the positions of the t.l.c. origin and PtdIns3*P* respectively.

protein-tyrosine kinases (reviewed by Cantley *et al.*, 1991). Although the role of tyrosine phosphorylation in the activation of these molecules is not conclusively established, these results have put forward new concepts in signal transduction involving the formation of multi-enzyme complexes.

We have previously examined the ability of p85α, p85β and PtdIns 3-kinase to associate with both the PDGF and EGF receptors after phosphorylation *in vitro* of the receptor itself (Otsu *et al.*, 1991). It was observed that, although the PtdIns 3-kinase complex bound specifically to the PDGF, but not the EGF, receptor, the two free p85 subunits could bind equally well to either receptor. Co-expression experiments with a variety of protein-tyrosine kinases were used in the present study to assess their interactions with the p85 proteins *in vivo*. It should be noted that in insect cells expressing recombinant growth-factor receptors it is not possible to stimulate their protein-tyrosine kinase activity with the respective growth factor, as they appear to be constitutively active (Greenfield *et al.*, 1988; R. Dhand, unpublished work). Therefore it was not necessary to add growth factors in our assays to obtain the observed effects.

Our results demonstrate that, when co-expressed, both p85α and p85β proteins complex with, and are phosphorylated by, activated growth-factor receptors and cytoplasmic protein-tyrosine kinases. Essentially identical results were observed with either p85α or p85β, suggesting that the p85 subunits themselves are probably not sufficient to reconstitute the specificity of binding observed with the active PtdIns 3-kinase (Otsu *et al.*, 1991). The ability of recombinant, baculovirus-expressed p85 proteins to block binding of the bovine brain PtdIns 3-kinase to the CSF-1 receptor demonstrates that both the PtdIns 3-kinase complex and these recombinant p85 proteins bind to a similar region of the CSF-1 receptor. A similar observation has been made by Escobedo *et al.* (1991), who demonstrated that the

murine p85α homologue can compete with PtdIns 3-kinase activity in NIH 3T3 cell lysates for binding to PDGF receptor phosphorylated *in vitro*.

The generation of antibodies against the p85 proteins has facilitated the further characterization of the PtdIns 3-kinase complex. One question of great importance is the mechanism by which the PtdIns 3-kinase is activated after ligand stimulation of a receptor. We demonstrate here that PtdIns 3-kinase activity can be immunoprecipitated from a number of cell lines by using the p85α antibody, both before and after growth-factor additions. Stimulation of responsive cells with PDGF causes a 20–50-fold-increase in associated PtdIns 3-kinase activity (Fig. 6; Kaplan *et al.*, 1987). However, it is not clear whether the subsequently observed increase in cellular 3-phosphorylated phosphoinositides results from the recruitment of this enzyme to a receptor-bound subcellular location where it has access to its substrates, or whether an increase in the activity of the PtdIns 3-kinase also occurs after binding to receptor or phosphorylation of components of the PtdIns 3-kinase complex on tyrosine residues. The data in Fig. 6 suggest that, although there may be a small increase in the total cellular PtdIns 3-kinase activity (2–3-fold), this probably does not account for the large increases in activity observed to be associated with PDGF receptor. This suggests that the SH2-domain-mediated association with the receptor (Otsu *et al.*, 1991) is probably the most important step in the generation of the 3-phosphorylated phosphoinositides observed after PDGF stimulation of cells (Auger *et al.*, 1989). It cannot, however, be ruled out that activation of this enzyme, i.e. by tyrosine phosphorylation, also has a role to play in cell-signalling mechanisms. This is currently an area of intense study.

Only trace amounts of PtdIns 3-kinase activity were detected in immunoprecipitations from either cell lines or bovine brain PtdIns 3-kinase preparations by using p85β antibody. Preliminary immunoblotting experiments, however, show that the bovine brain PtdIns 3-kinase preparation lacks detectable p85β and that there are only low levels (less than 10% relative to p85α) of p85β in the cell lines immunoprecipitated in this study (results not shown). Immunoblotting of rat tissues suggests that brain is the major site of p85β expression (M. J. Fry, unpublished work). The data in Fig. 4 suggest that there may be a small pool of p85β-associated PtdIns 3-kinase activity and that p85b may be an alternative regulatory subunit of the PtdIns 3-kinase complex. However, this result requires substantiation in other cell lines. The identification of suitable cell lines expressing endogenous p85β will be required before we are able to address fully whether this protein plays a cell-type-specific role in forming complexes with the PtdIns 3-kinase, or whether it is a regulatory subunit for some other cellular activity.

The results presented here show that the two recombinant p85 proteins possess most of the observed properties of the endogenous cellular p85. They are good substrates *in vivo* and *in vitro* for all protein-tyrosine kinases thus far examined, including members of both the receptor and non-receptor classes. They also associate with these protein-tyrosine kinases when co-expressed, and this association can be reconstituted *in vitro* in a manner dependent on tyrosine phosphorylation of the protein-tyrosine kinase. These observations further demonstrate the importance of SH2 domains in mediating these interactions and their role in coupling protein-tyrosine kinases to important intracellular second-messenger systems such as the PtdIns 3-kinase. From our data, we favour a model for PtdIns 3-kinase activation whereby the SH2-domain-containing p85 proteins serve as adaptors that allow the placement of the catalytic subunit near the plasma membrane, where its substrates are found and where it may be directly activated by phosphorylation or other mechanisms. It is not clear at the moment what the role of tyrosine phosphorylation of the p85 proteins is, but it could be involved in such activation events.

Baculoviral expression vector pAcC4 and wild-type *Autographa californica* nuclear polyhydrosis virus (AcNPV) were provided by Dr. M. Summers. The $p59^{c\text{-}fyn}$ baculovirus and an anti-peptide serum against this protein, together with the anti-PR4 serum, were kindly provided by Dr. S. A. Courtneidge (EMBL, Heidelberg, Germany). The cDNA encoding the human CSF-1 receptor and antibodies against this receptor were kindly given by Dr. C. Sherr (St. Jude Hospital, Memphis, TN, U.S.A.). We also thank Alistair Sterling and Oanh Nguyen for oligonucleotide and peptide synthesis respectively.

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